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BRIEF REPORT

Fusobacterium nucleatum supresses anti-tumor immunity by activating CEACAM1

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ABSTRACT

Fusobacterium nucleatum (*F. nucleatum*) is an oral anaerobe found to be enriched in colorectal cancer (CRC). Presence of *F. nucleatum* in CRC has been correlated with resistance to chemotherapy and poor prognosis. We previously demonstrated that the Fap2 outer-surface protein of *F. nucleatum* binds and activates the human inhibitory receptor TIGIT which is expressed by T and Natural Killer (NK) cells, and inhibits anti-tumor immunity. Here we show that *F. nucleatum* also binds and activates the human inhibitory receptor CEACAM1 leading to inhibition of T and NK cells activities. Our results suggest that using CEACAM1 and TIGIT inhibitors and specific targeting of fusobacteria should be considered for treating fusobacteria-colonized tumors

Introduction

F. nucleatum is a Gram-negative, non-spore forming anaerobe involved in the development of periodontal disease.[1] Interestingly, *F. nucleatum* was found to be overabundant in colorectal cancer (CRC),[2,3] where it accelerates tumor progression,[4,5] and is associated with poor outcome.[6] *F. nucleatum* potentiates colon tumorigenesis by modulation to a tumor-favorable immune microenvironment,[4] activation of β-catenin signaling,[7] silencing of tumor suppression genes,[8] and promotion of CRC resistance to chemotherapy.[9]

The killing activity of practically all immune cells is controlled by inhibitory and activating receptors. We have previously discovered that the *F. nucleatum* Fap2 outer surface protein binds and activates the TIGIT inhibitory receptor.[10] TIGIT is expressed on T and NK cells,[11] and its activation by tumor colonized fusobacteria is therefore suggested to protect the tumor from killing by the immune cells.[10]

CEACAM1 is a member of the carcinoembryonic antigen-related cell adhesion molecules (CEACAMs). CEACAM1 also serves as an inhibitory receptor on various immune cell subsets.[12,13] CEACAM1 has several ligands; it binds to itself and also to the Opa (Opacity-associated proteins) protein of pathogenic *Neisseria*. Activation of CEACAM1 by CEACAM1 leads to the inhibition of immune cell activities, whereas the Opa activation of CEACAM1 impairs normal maturation of immature dendritic cells, suppresses lymphocyte responses to activating stimuli, and also impairs phagocytic engulfment of the bacteria.[13] CEACAM1 also binds HopQ which enables CagA translocation[14-16] and also protects tumors from immune cell attack.[17]

CEACAM1 is expressed on the surface of a wide variety of tumors, and is considered to be a specific biomarker associated with tumor progression, metastasis and poor prognosis.[18] Specifically, it was previously shown that CEACAM1 expression is higher with more advanced stages of CRC, particularly in metastatic colon cancer, suggesting a role in CRC progression.[19] Importantly, Zhang et al.,[20] have shown that CEACAM1 is also highly expressed on circulating CD8+ T cells in CRC patients and elevated on Tumor Infiltrating Lymphocytes (TILs) compared with paraneoplastic T cells. When isolated T cells from tumor and paraneoplastic tissues were stimulated with anti-CD3 and subsequently analysed for IFN-γ secretion, CEACAM1+CD8+T cells derived from the CRC tissue, produced significantly less IFN-γ when compared with para-cancer tissue,[20] which implies CEACAM1's role in mediating T cell exhaustion.[21] Studies with other cancers displayed increased expression of CEACAM1 on CD4+, CD8+TILs, and on intra-tumoral NK cells, and decreased IFN-γ secretion from TILs and NK cells was also observed.[22-24]

In this study, we show that *F. nucleatum* binds and activates CEACAM1 leading to the inhibition of immune cell activities suggesting its role in tumor progression.

Results

F. nucleatum directly interacts with human CEACAM1

In order to investigate whether *F. nucleatum* interacts with CEACAM1, we used a reporter system that we had previously generated,[10,25] in which murine thymoma BW cells are transfected with chimeric proteins composed of the extracellular portion of an inhibitory or activating receptor fused to the mouse zeta chain. In this reporter system mouse IL-2 is secreted if the receptor is bound and activated by specific ligands. We used the parental BW cells and BW cells expressing either human activating receptors (NKp44, NKp30,

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CD16, 2B4 and DNAM1) or human inhibitory receptors (CEACAM1, and TIGIT), and incubated them with *F. nucleatum* ATCC 23726 (FN726). As can be seen in Figure 1(a), both TIGIT and CEACAM1 expressing BW cells were activated by *F. nucleatum*, as IL-2 secretion was detected only when these two reporter cells were used.

We further used the BW and BW-CEACAM1 cells (Figure 1 (b)) to demonstrate a direct interaction between CEACAM1 and *F. nucleatum*. We labeled *F. nucleatum* with FITC and then tested whether the labelled bacterium can interact with CEACAM1. As can be seen in Figure 1(c), an increased, dose-dependent binding of *F. nucleatum* to BW-CEACAM1 cells was observed. We then tested the reporter systems BW-TIGIT (Figure 1(d)) and BW-CEACAM1 (Figure 1(e)) against the wild type FN726 and its mutant strain K50 (FNK50), in which Fap2 (the fusobacterial ligand for the TIGIT receptor) has been inactivated[10]. While

activation of BW-TIGIT was Fap2-depenent (Figure 1(d)), activation of BW-CEACAM1 was not (Figure 1(e)).

We further used ELISA plates which were coated with either the FN726 or with its Fap2-deficient mutant strain FNK50. We then incubated the human CEACAM1-Ig, and the TIGIT-Ig fusion proteins with the immobilized bacteria. As can be seen, while the binding of TIGIT to *F. nucleatum* is indeed Fap2-dependent (Figure 1(f)), the human CEACAM1-Ig binding is not (Figure 1(g)). In order to corroborate our results, we used human, rat or mouse CEACAM1-Ig fusion proteins in which the extracellular domain of CEACAM1 is fused to the Fc portion of human IgG1. As a control, we used 2B4-Ig (Control-Ig fusion protein) that was prepared in a similar manner. As can be seen in Figure 1(h), binding of human CEACAM1-Ig was observed to FN726. No binding was observed when 2B4-Ig was used, or when CEACAM1-Ig
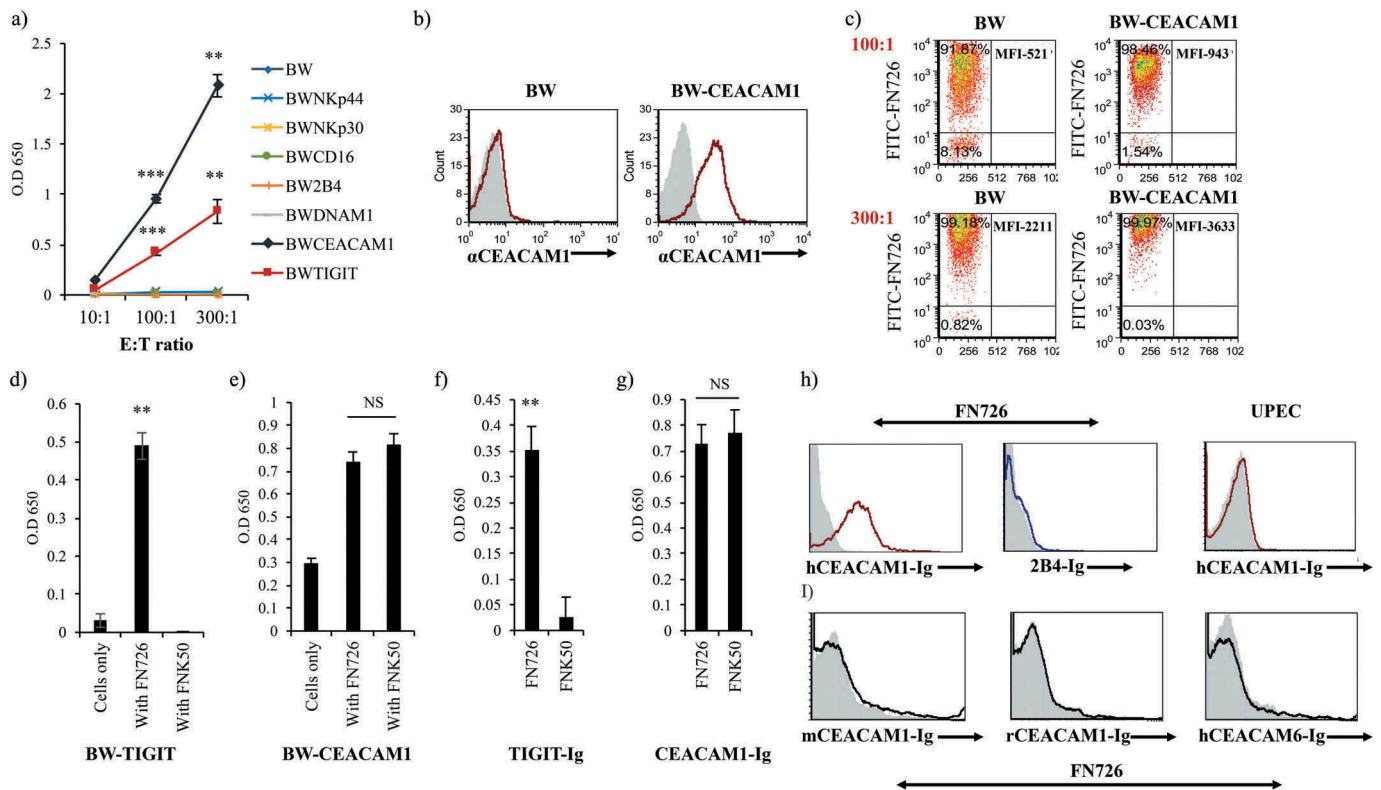

Figure 1. CEACAM1 interacts with *Fusobacterium nucleatum*.

(a) Parental BW cells, and BW cells expressing human NK cell activating receptors: NKp44, NKp30, 2B4, DNAM1, and CD16, or human inhibitory receptors: CEACAM1 and TIGIT fused to mouse zeta chain, were incubated with FN726. The bacteria to BW ratios were 10:1, 100:1, and 300:1 (as indicated at the X axis). The presence of mouse IL-2 in the supernatants was determined by ELISA 48hr later. The figure shows one representative experiment out of three performed. The error bars are derived from triplicates. **p < 0.005, ***p < 0.0005. Bonferroni corrected two tailed paired t test (differences between paired values). (b) Flow cytometry of BW (left) and the same cells transfected to express CEACAM1 as indicated (right). Cells were stained with anti-human CEACAM1 mAb (red empty histograms) or with secondary control antibodies only (grey filled histograms). (c) Flow cytometry of binding of FITC-labelled FN726 to parental BW (left dot plots), and BW cells expressing the human CEACAM1 receptor (right dot plots). The bacteria to targets (BW or BW-CEACAM1) ratios are indicated in the Y axis. The Median Fluorescent Intensity (MFI) of the FITC-labelled FN726 binding to the target cells are indicated in the dot plots. One representative plot out of duplicate is shown. One experiment out of two performed is presented. *p < 0.05 for 300:1, **p < 0.005 for 100:1. (D-E) The TIGIT expressing BW cells (d), and the CEACAM1 expressing BW cells (e), were incubated with the parental FN726 or its FAP2 deficient mutant FNK50 at 100:1 bacteria to BW-TIGIT or BW-CEACAM1 ratio. Mouse IL-2 in the supernatants was determined by ELISA 48hr later. Figure shows one representative experiment out of three performed. **p < 0.005. Two tailed paired t test (differences between paired values). (f-g) 0.5 × 10⁶ FN726, and the Fap2 mutant K50 (FNK50), were bound in wells of ELISA plates. Plates were incubated with human TIGIT-Ig (h) or with human CEACAM1-Ig (i), and ELISA assays were performed. Figure shows one representative experiment out of three performed. The error bars are derived from triplicates. **p < 0.005. Two tailed paired t test (differences between paired values). (h) FN726 was stained with 1µg of human CEACAM1-Ig (hCEACAM1, left) and 2B4-control-Ig (middle). Irrelevant bacterium, Urinary Pathogenic *E. coli* (UPEC) CFT 073 was stained with 1µg of hCEACAM1-Ig (right). (i) FN726 was stained with 1µg of mouse CEACAM1-Ig (mCEACAM1, left) rat CEACAM1-Ig (rCEACAM1, middle), and hCEACAM6-Ig (right). Empty colored histograms represent the specific staining, and the filled grey histograms represent staining of secondary control antibodies only. One representative staining out of three is shown.

was incubated with irrelevant bacteria Urinary Pathogenic *E. coli* CFT073 (UPEC). Importantly, no binding of mouse or rat CEACAM1-Ig was observed to FN726 (Figure 1(i), left and middle, respectively), or when using other CEACAM-Ig's e.g CEACAM6-Ig (Figure 1(i), right). Taken together, using four different assays we successfully demonstrated that *F. nucleatum* interacts with CEACAM1.

F. nucleatum inhibits T cells and NK cells function

To test whether the binding of *F. nucleatum* to CEACAM1 can inhibit T cell function, we isolated CD4+ and CD8+ T cells from peripheral blood of healthy donors and cultivated them in IL2 containing medium as described in "Materials and Methods". The growth medium activated the T cells and subsequently enabled the selection of T cells which highly express CEACAM1 (Figure 2(a)).

We next wanted to assess whether *F. nucleatum* affects their function and employed "redirected cytokine and cytotoxic assays". These assays required the use of mouse mastocytoma P815 cells which express the FcγR. These cells were then incubated with an anti-CD3 antibody which bound the FcγR via its Fc portion. The bound anti-CD3 could then activate the T cells via CD3 and induce the secretion of IFN-γ or CD107a degranulation.

For the redirected cytokine assays, P815 cells were initially incubated either alone, with anti-CD3, or with anti-CD3 together with *F. nucleatum* or the Fap2 mutant FNK50.
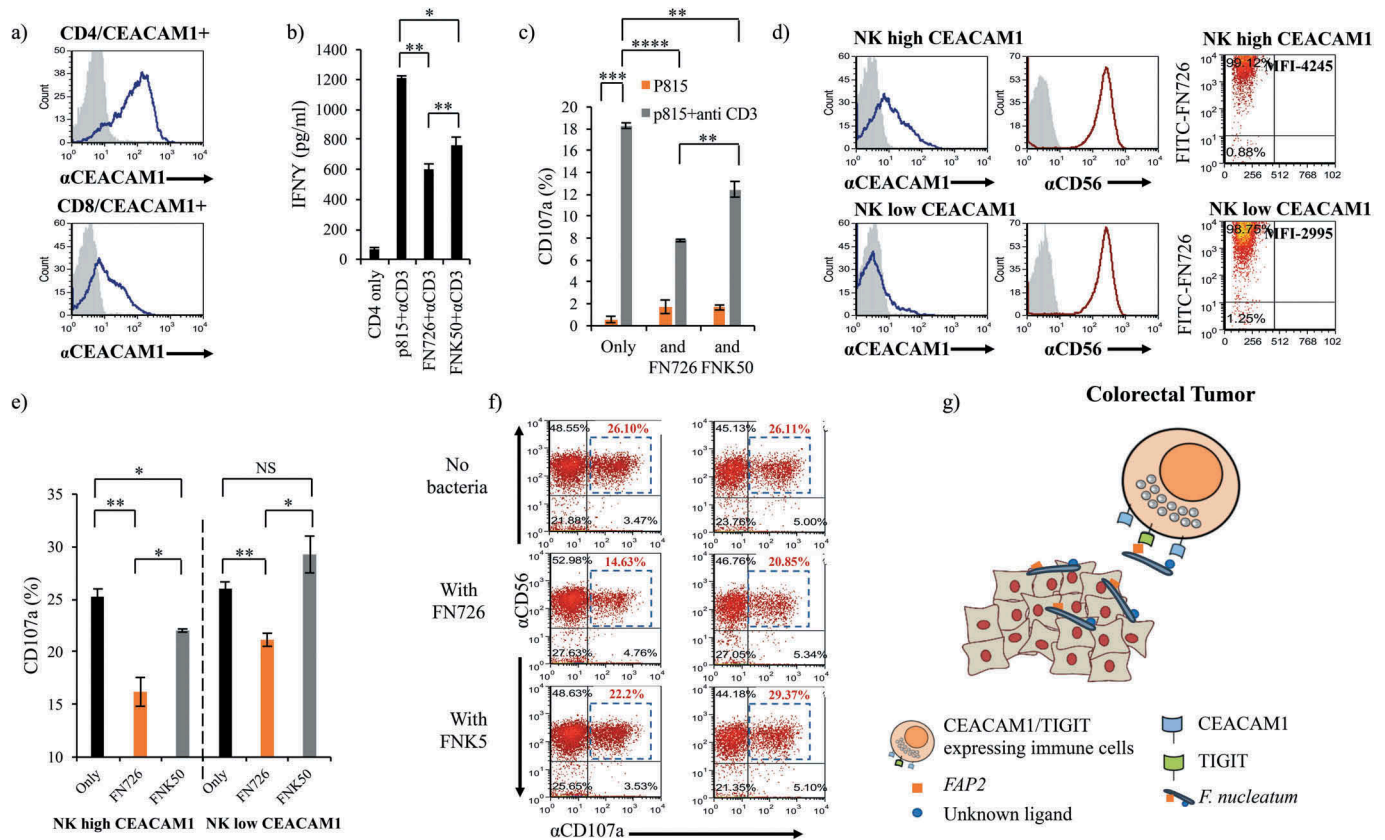

Figure 2. *Fusobacterium nucleatum* inhibits immune cells activity via CEACAM1 activation.

(a) Flow cytometry of representative primary IL-2 activated human CD4+ T cells (upper), or CD8+ T cells (lower), expressing CEACAM1, stained with anti-CEACAM1 mAb (blue empty histograms), or with secondary control antibodies only (grey filled histograms). (b) The murine FcγR-positive mastocytoma P815 cells were pre-incubated with anti-CD3 mAb (0.2μg/well), and then incubated with or without WT FN726 or with K50 (as indicated in the x axis) at bacteria to P815 ratio of 50:1. IL-2 activated CD4-expressing cells, expressing CEACAM1 (indicated in A) were added and incubated with the P815 cells in an E:T ratio of 1:1. The presence of the IFN-γ in the supernatants was determined by ELISA 48 hr later (represented by pg/ml). The error bars are derived from triplicates. *p =0.011, **p < 0.005. Bonferroni corrected one tailed paired t test (differences between values). The figure represents data collected from two independent experiments. (c) CD8 cytotoxicity was assayed by CD107a degranulation assay. CD8 cells (25000/w) expressing the CEACAM1 receptor (indicated in A), were incubated with the murine FcγR-positive mastocytoma P815 cells pre-coated with anti CD3 mAb (0.2μg/well) with or without FN726 or with FNK50 at target (P815) to bacteria ratio of 300:1. The effector (CD8) to target (P815) ratio was 1:1. The Y axis indicates the percentages of CD3-mediated redirected killing (represented as CD107a+) of the P815 cells by CEACAM1 positive CD8 cells. Data are shown as means percentage and ±SD of three replicates. **p < 0.005, ***p < 0.0005, ****p < 0.0001. Bonferroni corrected two tailed paired t test (differences between paired values). The figure represents data collected from two independent experiments. (d) Flow cytometry of representative primary IL-2 activated human NK cells expressing high levels or low levels of CEACAM1(upper and lower histograms, respectively), stained with anti-CEACAM1 mAb (blue empty histograms), anti CD56 (red empty histograms) or with secondary control antibodies only (grey filled histograms). Right panels indicate staining of NK cells expression high (upper dot plot) or low (bottom dot plot) CEACAM1 with FITC labelled FN726. The Median Fluorescent Intensity (MFI) of the FITC-labelled FN726 binding to the target cells are indicated in the dot plots. (e) NK cells (25000/w) expressing high levels (left) or low levels (right) of CEACAM1 receptor (indicated in D), were incubated with 721.221 cells that were pre-coated with the WT FN726 or with FNK50, at effector (NK) to bacteria ratio of 300:1. The effector to target (721.221) ratio was 1:1. The Y axis represents the percentages of killing of the 721.221 cells by the NK cells (represented as CD107a+). Data are shown as means percentage and ±SD of three replicates. *p < 0.05, **p < 0.01, Bonferroni corrected One tailed paired t test (differences between paired values). The figure represents data collected from two independent experiments. (f) Flow cytometry dot plots of representative CD107a degranulation assay with NK cells expressing high (left) or low (right) CEACAM1 in the presence of the WT FN726 or the Fap2 mutant FNK50. The percentages of CD107a degranulation are shown in the dot plots. (g) Schematic representation of the suggested involvement of FN726 in immune cells modulation in colorectal tumor.

Subsequently, the CEACAM1-positive CD4⁺ T cells were added. IFN-γ secretion was analysed 48 hours later. As can be seen in Figure 2(b), in the presence of anti-CD3 mAb, there were high levels of IFN-γ. However, in the presence of FN726 or FNK50, the IFN-γ secretion was significantly inhibited. The inhibition was more prominent in the FN726, that activates both TIGIT (in a Fap2-dependent manner) and CEACAM1 (in Fap2-independent manner), compared with the Fap2 mutant FNK50.

We next tested the CEACAM1-positive CD8⁺ T cells in a "redirected cytotoxic assay" using the CD107a degranulation assays (Figure 2(c)). Previous studies have shown that the presence of CD107a⁺ correlates well with cytotoxic activity of CD8⁺ T cells.[26] Following incubation of P815 cells with anti-CD3 mAb, we coated the cells with or without the FN726 and its Fap2 mutant FNK50, and then co-cultured the P815 cells with CEACAM1⁺ CD8+ T cells, and stained the CD8⁺ T cells for CD107a.

As shown in Figure 2(c), anti-CD3 indeed induced marked redirected degranulation of the P815 cells by the CD8 CEACAM1⁺ cells. However, in the presence of FN726, this redirected degranulation was significantly inhibited. When the Fap2 mutant FNK50 was used, inhibition was still observed, but it was significantly less than the WT bacteria.

Lastly, we also investigated whether CEACAM1 activation by FN726 can inhibit NK cell cytotoxicity. As in T cells, CEACAM1 is expressed on NK cells following activation and is mainly expressed on the CD16-negative subset.[23,24] We therefore isolated CD16-negative NK cells, and activated them with IL-2. We used two NK cells expressing high and low levels of CEACAM1 (Figure 2(d), upper and lower panels, respectively), where the low expressing NK cells are bulk NK cells. We observed an increased binding of FITC labelled F. nucleatum to the NK cells expressed high levels of CEACAM1 compared to the NK cells expressed low levels (Figure 2(d), dot plots). We then performed the CD107a degranulation assay using 721.221 cells as targets. In the presence of the FN726 and NK cells expressing high levels of CEACAM1, killing was significantly inhibited (Figure 2(e), left, and representative dots plot in F, left, middle). In the presence of the Fap2 mutant FNK50, inhibition was still observed (probably due to CEACAM1 activation by F. nucleatum), as with the CD8⁺ cells, but the inhibition was significantly lower than the WT fusobacteria (apparently because the Fap2 mutant cannot engaged TIGIT, which expressed on all NK cells), (Figure 2(e), left, and representative dots plot in F, left, lower). Inhibition of degranulation was also observed when the WT F. nucleatum and bulk NK cells that express low levels of CEACAM1 were used (Figure 2(e), right, and representative dots plot in F, right). This was probably due to TIGIT engagement by FN726 since no inhibition was observed when the Fap2 mutant FNK50 was used (Figure 2 (e), right, and representative dots plot in F, right, lower). Taken together, maximal effect of degranulation inhibition was seen when both the CEACAM1 and the TIGIT inhibitory receptors were activated by the unknown CEACAM1 and Fap2 bacterial ligands, respectively (a summary model is presented in Figure 2(g)).

Discussion

We have previously demonstrated that the colon cancer associated bacterium, F. nucleatum inhibits antitumor immunity via the binding of the fusobacterial Fap2 protein to the TIGIT inhibitory receptor.[10] We also demonstrated recently[14,17] that another bacterium associated with cancer, H. pylori also inhibits immune cell activity via the binding of its HopQ protein to CEACAM1. Here we show that in addition to TIGIT, F. nucleatum also binds CEACAM1 to inhibit immune cell activity.

Previous studies[20] have shown that TILs in CRC expressed high levels of CEACAM1 compared with paraneoplastic T cells, and produced significantly less IFN-γ compared with T cells derived from para-cancer tissue. The higher CEACAM1 expression on CRC TILs suggests that CEACAM1 play a role in mediating T cell exhaustion.[20]

We have shown in this study that F. nucleatum, a bacteria associated with tumor promotion and acceleration, binds and activates the inhibitory receptor CEACAM1, indicating its potential importance in modulating anti-tumor immunity. The interaction of the unknown F. nucleatum ligand with CEACAM1 on immune cells, in addition to the homophilic interactions of CEACAM1, occurring between CEACAM1⁺ TILs, and CEACAM1⁺ tumor cells, may help cancer evade immune cell attack in an additive mechanism.

Search in the FN726 genome for homologues of HopQ did not yield a promising candidate. The F. nucleatum protein that binds CEACAM1 is currently unknown, but is different from the one used by the bacterium to bind and activate TIGIT.[10] It seems that the different ligands have an additive effect as the inhibition of killing and IFN-γ secretion in the presence of the F. nucleatum FN726 is significantly stronger compared with the Fap2 mutant K50 (that is impaired in TIGIT activation).

It will be interesting to determine in the future the identity of the F. nucleatum protein that interacts with CEACAM1.

Materials and methods

Primary human T cells, NK cells, cell lines, fusion proteins and antibodies

Primary human NK cells were isolated from PBLs of healthy donors (providing informed consent), using the EasySep™ human NK cell enrichment kit (StemCells Technologies). NK cell purity was 100% as determined by positive CD56 and NKp46 expression and negative CD3 expression. CD16⁻ NK cells were isolated in negative selection using the human APC selection kit (STEMCELL Technologies). Isolated NK cells negative for CD16 and positive for CEACAM1 were activated with IL-2. Primary human CD4⁺ and CD8⁺ cells were isolated from PBLs of healthy donors. Cells positive for CEACAM1 were pooled together and grown in the presence of IL-2. Activated NK and T cells were generated by culturing isolated NK and T cells together with irradiated feeder cells (2.5×10^4 allogeneic PBMCs from two donors and 5×10^3 RPMI8866 cells in each well) and 20 μg ml PHA (Roche, Rehovot, Israel). Both PBMCs and RPMI8866 cells were irradiated in 6000 rad before seeding in 96-well U-bottom plates. The cultures were maintained in

DMEM:F-12 Nutrient Mix (Sigma Aldrich, Rehovot, Israel; 70:30), 10% human serum (Sigma Aldrich), 2 mM glutamine (Biological Industries; BI, Beit-Haemek, Israel), 1 mM sodium pyruvate (BI), 1×nonessential amino acids (BI), 100 U ml penicillin (BI), 0.1 mg ml streptomycin (BI) and 500 U ml rhIL-2 (Peprotech, Rehovot, Israel). A week later, irradiated feeder cells (2.5 × 10^4 allogeneic PBMC from two donors and 5 × 10^3 RPMI8866 cells in each well) were added.

The following cell lines were used: the mouse thymoma BW cells, the human EBV transformed 721.221 cells, and the murine lymphoblastlike mastocytoma p815 cell line. The generation of the BW-CEACAM1 transfectant was described previously.[10] The generation of CEACAM1-Ig, and the Control-Ig fusion proteins was previously described.[10,14]

Bacterial growth conditions

F. nucleatum ATCC 23726 (FN726), and *F. nucleatum* K50, were grown in Wilkins Chalgren broth (Oxoid, UK) in an anaerobic chamber (Bactron I-II Shellab, USA) with an atmosphere of 90% N2, 5% CO_2 and 5% H_2 at 37°C. Thiamphenicol (2.5 µg/ml) was added for growth of K50. Uropathogenic *Escherichia coli* CFT073 was grown in LB broth (Difco, USA) under aerobic conditions at 37°C.

BW assays

The various bacteria were placed in 96 well plates and incubated for one hour at 37°C in medium containing RPMI supplemented with 10% FCS and penicillin-streptomicin. Subsequently, cells (50,000 of the appropriate BW or BW transfectants) were added and incubated together with the bacteria for 48 hours at 37°C, 5% CO_2. The final cell to bacteria ratios is indicated in the figure legend. Next, supernatants were collected and the presence of mouse IL-2 in the supernatants was determined using standard ELISA assay.

FITC labelling of bacteria and binding to cells

F. nucleatum (~10^9 CFU/ml) was labelled with fluorescein isothiocyanate (FITC) (0.1 mg/ml in PBS; Sigma-Aldrich) for 30 min at room temperature and washed three times in PBS. FITC labelled bacteria were incubated with various cells at various bacteria to cell ratios for 30 minutes at 4°C. Cells were washed and bacterial binding was detected using flow cytometry.

Degranulation and cytokine secretion assays

For the redirected cytokine or CD107a degranulation assays with T cells, 25000/w of P815 cells were pre-coated for 1 hour on ice with 0.2 µg per well of the anti-CD3 mAb. Then, for the redirected cytokine secretion assay, P815 cells (that were pre-coated with anti-CD3 mAb and co-incubated with bacteria at bacteria to target ratio of 50:1) were added to CEACAM1 positive CD4+ cells. The E:T ratio was 1:1. Levels of IFN-γ in supernatants were measured 48 hours later by ELISA using matching antibodies against IFN-γ (pair BLG-502402, 502504). For the redirecting CD107a degranulation assay with CD8, bulk CD8 cells positive for CEACAM1

were added and incubated for 2 hours at 37°C with P815 cells that were pre-coated with anti-CD3 mAb and bacteria (at bacteria to target ratio of 300:1) in the presence of 0.1 µg allophycocyanin-conjugated CD107a mAb (Biotest, Ness Ziona, Israel) for 2 h at 37 °C. CD107a levels on the bulk CD8 cells positive for CEACAM1 cells were determined by flow cytometry.

For NK cell CD107a degranulation assay, 25000/well of NK cells were incubated with 721.221 cells that were pre-coated with bacteria (at bacteria to target ratio of 300:1) in a E:T ratio of 1:1 in the presence of 0.1 µg allophycocyanin-conjugated CD107a mAb (Biotest, Ness Ziona, Israel) for 2 h at 37°C. CD107a levels on the NK cells were determined by flow cytometry.

Statistical analysis

GraphPad Prism software version 6.0 was used for statistical analysis. Statistical tests used are indicated in the figure legends.

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